



PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 15/00		A1	(11) International Publication Number: WO 91/07487
			(43) International Publication Date: 30 May 1991 (30.05.91)
(21) International Application Number: PCT/US90/06597			(74) Agents: SIBLEY, Kenneth, D. et al.; Bell, Seltzer, Park & Gibson, P.O. Drawer 34009, Charlotte, NC 28234 (US).
(22) International Filing Date: 13 November 1990 (13.11.90)			
(30) Priority data: 437,848 16 November 1989 (16.11.89) US			(81) Designated States: AT, AT (European patent), AU, BB, BE (European patent), BF (OAPI patent), BG, BJ (OAPI patent), BR, CA, CF (OAPI patent), CG (OAPI patent), CH, CH (European patent), CM (OAPI patent), DE, DE (European patent), DK, DK (European patent), ES, ES (European patent), FI, FR (European patent), GA (OAPI patent), GB, GB (European patent), GR (European patent), HU, IT (European patent), JP, KP, KR, LK, LU, LU (European patent), MC, MG, ML (OAPI patent), MR (OAPI patent), MW, NL, NL (European patent), NO, RO, SD, SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent).
(71) Applicants: DUKE UNIVERSITY [US/US]; Erwin Road, Durham, NC 27706 (US). CORNELL RESEARCH FOUNDATION, INC. [US/US]; 20 Thornwood Drive, Suite 105, Ithaca, NY 14850 (US). E.I. DU PONT DE NEMOURS & COMPANY (INC.) [US/US]; 1008 Market Street, Wilmington, DE 19898 (US).			
(72) Inventors: JOHNSTON, Stephen, A. ; 309 North Gregson Street, Durham, NC 27701 (US). WILLIAMS, R., Sanders ; 406 Watts Street, Durham, NC 27701 (US). SANFORD, John, C. ; 43 Sunset Drive, Geneva, NY 14456 (US). McELLYGOTT, Sandra, G. ; 27 South Longpoint Lane, Rose Valley, PA 19063 (US).			Published With international search report.
(54) Title: PARTICLE-MEDIATED TRANSFORMATION OF ANIMAL TISSUE CELLS			
(57) Abstract			
<p>A method of transferring a gene to vertebrate cells is disclosed. The method comprises the steps of: (a) providing microprojectiles, the microprojectiles carrying polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the tissue cells and a gene positioned downstream of the regulatory sequence and under the transcriptional control thereof; and (b) accelerating the microprojectiles at the cells, with the microprojectiles contacting the cells at a speed sufficient to penetrate the cells and deposit the polynucleic acid sequences therein. Preferably, the target cells reside <i>in situ</i> in the animal subject when they are transformed. Preferred target cells are dermis or hypodermis cells, and preferred genes for insertion into the target cells are genes which code for proteins or peptides which produce a physiological response in the animal subject.</p>			

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FI	Finland	ML	Mali
AU	Australia	FR	France	MN	Mongolia
BB	Barbados	GA	Gabon	MR	Mauritania
BE	Belgium	GB	United Kingdom	MW	Malawi
BF	Burkina Faso	GN	Guinea	NL	Netherlands
BG	Bulgaria	GR	Greece	NO	Norway
RJ	Benin	HU	Hungary	PL	Poland
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	SD	Sudan
CF	Central African Republic	KP	Democratic People's Republic of Korea	SE	Sweden
CG	Congo	KR	Republic of Korea	SN	Senegal
CH	Switzerland	LI	Liechtenstein	SU	Soviet Union
CI	Côte d'Ivoire	LK	Sri Lanka	TD	Chad
CM	Cameroon	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark	MG	Madagascar		
ES	Spain				

-1-

PARTICLE-MEDIATED TRANSFORMATION
OF ANIMAL TISSUE CELLS

Summary of the Invention

This invention relates to the transformation of animal cells and tissue with heterologous DNA by microprojectile bombardment.

5

Background of the Invention

The transformation of living cells by propelling microprojectiles at those cells at high velocity, with the microprojectiles carrying exogenous DNA or RNA, was originally proposed by T. Klein, E. Wolf, R. Wu and J. Sanford, Nature 327, 70 (1987). See also J. Sanford et al., Particulate Science and Technology 5, 27 (1987). The original work involved the transformation of onion epidermal cells with RNA derived from tobacco mosaic virus. The findings with onion epidermal cells have been extended to other plants. For example, the transformation of soybean callus by particle bombardment is described by P. Christou et al., Plant Physiol. 87, 671 (1988), and the transformation of soybean meristem is described by D. McCabe et al., Bio/Technology 6, 923 (1988). See also B. Spalding, Chemical Week, 16 (Aug. 31, 1988); 20 European Patent Application Publication No. 0 301 749 to P. Christou et al. The transformation of embryonic maize callus cells by particle bombardment is described by T. Klein et al., Proc. Natl. Acad. Sci. USA 85, 4305 (1988), and the production

-2-

of transformed maize seed by the particle bombardment of maize pollen is described in European Patent Application Publication No. 0 270 356 to D. McCabe et al.

In addition to the transformation of plants, microprojectile bombardment has been used to transform cellular organelles. Mitochondrial transformation in yeast by particle bombardment is described by S. Johnston et al., Science 240, 1538 (1988), and chloroplast transformation in Chlamydomonas by particle bombardment is described by J. Boynton et al., Science 240, 1534 (1988).

The use of particle bombardment for the transformation of animal tissue or cells has received comparatively little attention. Sanford et al., Particulate Science and Technology 5, 27, 35-36 (1987), suggest the use of particle bombardment for human gene therapy, but do not suggest the tissue type or the developmental stage of tissue useful for carrying out such therapy. U. S. Patent Application No. 06/877,619, titled "Method for Transporting Substances Into Living Cells and Tissues and Apparatus Therefor," concerns the introduction of biological materials into cells by microprojectile bombardment. Suggested biological substances are stains such as fluorescent or radiolabeled probes, viruses, organelles, vesicles, proteins such as enzymes or hormones, and nucleic acids such as DNA and RNA. Suggested procedures include: (a) the particle bombardment of animal cells such as eggs, bone marrow cells, muscle cells, and epidermal cells at page 16, lines 5-6; (b) the particle bombardment of human tissue or other animal tissue such as epidermal tissue, organ tissue, and tumor tissue at page 16, lines 13-14; and (c) human gene therapy for sickle cell anemia by the particle-mediated transformation of bone marrow tissue at page 22, lines 8-9.

W. Brill, Particle Propulsion by Electric Discharge (Tape of Speech at AAAS meeting on Plant Molecular Biology/Genetic Engineering for Agriculture (VI) (January 1989), discusses the transformation of nematodes to correct a missing body wall myosin gene by particle bombardment. The

-3-

utility of transforming nematodes is, however, comparatively limited.

In view of the foregoing, an object of this invention is to provide new uses for the treatment of animals, particularly vertebrates, and their tissues and cells, by microprojectile bombardment.

A more particular object of this invention is to use microprojectile bombardment as a means for administering proteins or peptides to an animal subject.

Summary of the Invention

A first aspect of the present invention is a method of transferring a gene to preselected vertebrate cells. The method comprises the steps of, first, providing microprojectiles, the microprojectiles carrying polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the vertebrate cells and a heterologous gene positioned downstream of the regulatory sequence and under the transcriptional control thereof. The microprojectiles are then accelerated at the preselected cells, with the microprojectiles contacting the cells at a speed sufficient to penetrate the cells and deposit the polynucleic acid sequences therein (as used herein, the plural form of terms such as "cell," "microparticle," and "polynucleic acid sequence" is intended to encompass the singular).

A second aspect of the present invention is a method of transferring a gene to preselected vertebrate tissue. The method comprises the steps of, first, providing microprojectiles, the microprojectiles carrying polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the vertebrate tissue and a heterologous gene positioned downstream of the regulatory sequence and under the transcriptional control thereof. The microprojectiles are then accelerated at the preselected tissue, with the microprojectiles contacting the cells of the tissue at a speed sufficient to penetrate the cells and deposit the polynucleic acid sequences therein.

-4-

A third aspect of the present invention is a method of transferring a gene to a preselected tissue in situ in a vertebrate subject. The method comprises the steps of, first, providing microprojectiles, the microprojectiles carrying polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the vertebrate tissue and a heterologous gene positioned downstream of the regulatory sequence and under the transcriptional control thereof. The microprojectiles are then accelerated at the animal subject, with the subject positioned so that the microprojectiles contact the preselected tissue, with the microprojectiles contacting the cells of the tissue at a speed sufficient to penetrate the cells and deposit the polynucleic acid sequences therein.

The data disclosed herein provide the first demonstration of particle-mediated transformation of (a) vertebrate cells, (b) vertebrate tissue, and (c) vertebrate tissue in situ of which these applicants are aware.

Also disclosed herein is a method of administering a protein or peptide to a vertebrate subject. This method is based in part on our finding that vertebrate tissue transformed by particle bombardment is surprisingly free of callus formation, inflammation, and other defensive responses. Thus, proteins and peptides released from the transformed cells (by virtue of their being transformed) can circulate throughout the animal subject in which the cells reside, and cells which circulate in the animal subject (e.g., lymphocytes) have access to the transformed cells. In this method, target vertebrate tissue (preferably dermis or hypodermis tissue) is selected and microprojectiles provided. The microprojectiles carry polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the selected tissue and a gene positioned downstream of the regulatory sequence and under the transcriptional control thereof. The gene codes for a protein or peptide. The microprojectiles are then accelerated at the selected target tissue, with the microprojectiles contacting

-5-

the cells of the tissue at a speed sufficient to penetrate the tissue cells and deposit the polynucleic acid sequences therein to provide transformed tissue cells. The transformed tissue cells are then maintained in the animal subject, with the transformed tissue cells present in the subject in a number sufficient to produce a physiological response (e.g., an endocrine response, an immune response) to the protein or peptide coded for by the gene in the subject upon expression of the gene.

Brief Description of the Drawings

The present invention is explained in greater detail in the Examples, Detailed Description, and Figures herein, in which:

Figure 1 is a perspective view of a currently available microprojectile bombardment apparatus;

Figure 2 is a detailed view of the bombardment chamber shown in Fig. 1, with the stopping plate and animal chamber positioned for insertion;

Figure 3 is a perspective view of an animal chamber positioned in a bombardment chamber, with the animal chamber sealing plate positioned for insertion;

Figure 4 is a side sectional view of the apparatus shown in Fig. 3, and showing the paths of travel of the macroprojectile to the stopping plate and the microprojectiles from the stopping plate to the subject;

Figure 5 is a detailed view of a stopping plate and sealing plate, showing the macroprojectile after impact on the sealing plate and the path of travel of the microprojectiles to the sealing plate;

Figure 6 shows the persistent heat-inducibility of the firefly luciferase gene driven by the human HSP70 promoter after transformation of cultured skeletal myotubes by microprojectile bombardment; and .

Figure 7 shows peak luciferase activity of skin and ear one day after transfection by microprojectile bombardment.

-6-

Detailed Description of the Invention

As used herein, the term "tissue" means an aggregation of similarly specialized cells united in the performance of a particular function. The term "tissue cells" means cells
5 residing in a tissue. The term "cell" means a cell either residing in a tissue (i.e., "in situ") or removed from its tissue of origin (i.e., "in vitro").

Animal tissue cells can be bombarded in situ with respect to their tissue of origin, or separated from the
10 tissue and bombarded in vitro. The cells are preferably transformed in situ with respect to the tissue of origin. Tissue to be transformed can likewise be bombarded either in vitro or in situ with respect to the animal of origin or the animal in which the transformed tissue is to subsequently be
15 maintained, depending on the result sought. Preferably, the tissue is transformed in situ in the animal in which it is to be maintained.

Animal subjects to be treated by the method of the present invention are vertebrates, exemplary being fish,
20 reptiles, amphibians, birds, and mammals. Birds (e.g., chicken, turkey) and mammals are preferred, and mammals (e.g., horse, cow, sheep, pig, human) are most preferred. Vertebrate tissues and cells to be treated by the method of the present invention are of corresponding origin, with the origins of the
25 preferred and most preferred tissues and cells corresponding to the preferred and most preferred animals.

The present invention may be practiced on any vertebrate cell, subject to the proviso that cells which have been immortalized in cell culture or otherwise altered from
30 their native state are excluded. Thus, cells to be transformed in the present invention are cells in their naturally occurring state (i.e., primary cells), whether they reside in a tissue or exist free from a tissue in vitro. Cells which have been maintained in vitro for a time
35 sufficient and/or under conditions effective to cause them to lose the characteristics they possess in situ are excluded

-7-

from the group of cells with which the present invention is concerned.

Vertebrate cells to be treated by the method of the present invention are preferably differentiated cells, and most preferably terminally differentiated cells such as skin cells, hypodermis cells, muscle cells, nerve cells, pancreas cells, and liver cells. Exemplary skin cells include the basal cells and the cells of the dermis and hypodermis.

Vertebrate tissue to be treated by the method of the present invention is likewise preferably differentiated tissue, and most preferably terminally differentiated tissue such as skin tissue, hypodermis tissue, muscle tissue, nerve tissue, pancreas tissue, and liver tissue. Exemplary skin tissues include the basal cell layer, the dermis, and the hypodermis.

The polynucleic acid sequence carried by the microprojectile is a recombinant construct of a gene and a regulatory element. The construct may take any suitable form, such as a plasmid, a genomic viral DNA sequence such as a bovine papillomavirus vector, see E. Chen et al., 299 Nature 529 (1982), a retroviral RNA sequence, derivatives of the foregoing, and synthetic oligonucleotides. The DNA constructs, particularly the plasmids, are currently preferred. Preferred genes which may be used in the polynucleic acid sequence are those which code for a protein or peptide which produces a physiological response (preferably an endocrine response or an immune response) in the animal subject. The gene may be homologous or heterologous with respect to the animal to be transformed, or may be a modified version of a homologous gene.

Exemplary of genes which code for proteins or peptides which produce an endocrine response (i.e., a physiological response in the animal at a point sufficiently removed from the transformed tissue region to require that the protein or peptide travel through the circulatory or lymphatic system of the subject) are genes which code for Factor VIII:C, genes which code for plasminogen activators such as Tissue

-8-

Plasminogen Activator and urokinase, see, e.g., U.S. Patents Nos. 4,370,417 and 4,558,010, genes which code for growth hormones such as human or bovine growth hormone, genes which code for insulin, and genes which code for releasing factors such as Luteinizing Hormone Releasing Hormone. The disclosures of all references cited herein are to be incorporated herein by reference.

Exemplary of genes which code for proteins or peptides which produce an immune response (i.e., a response in which B and/or T lymphocytes activated by the protein or peptide are capable of traveling in the circulatory or lymphatic system of the subject to a site removed from the transformed tissue) are genes coding for subunit vaccines such as disclosed in U.S. Patent No. 4,857,634 to Minor et al. titled "Peptides Useful in Vaccination against Enteroviruses," U.S. Patent No. 4,738,846 to Rose et al. titled "Vaccine for Vesicular Stomatitis Virus," U.S. Patent No. 4,428,941 to Galibert et al. titled "Nucleotidic Sequence Coding the Surface Antigen of the Hepatitis B Virus, Vector Containing Said Nucleotidic Sequence, Process Allowing the Obtention Thereof and Antigen Obtained Thereby," and U.S. Patent No. 4,761,372 to Maas et al. titled "Mutant Enterotoxin of E. coli."

An advantage of administering a protein or peptide capable of producing an immune response in the manner described herein is the ability to cause the immunogen to be effectively presented to the subject over an extended period of time. This is in contrast to the simple injection of a protein or peptide, which tend to be rapidly digested and cleared by the subject.

Exemplary of other genes which code for proteins or peptides which produce a physiological response in the subject include genes coding for enzymes such as α_1 antitrypsin, genes which code for receptors such as the insulin receptor, see U.S. Patent No. 4,761,371 to Bell et al., genes which code for adhesions such as the CD4 receptor, see EPO Patent Application Publication No. 0 314 317 of Genentech, titled "Adhesion variants, nucleic acid encoding them and compositions

-9-

comprising them," which may have therapeutic activity in the subject, genes which code for proteins or peptides which will either affect neighboring tissue cells (a paracrine-like action) or will be secreted and affect the secreting cell (an autocrine-like action), and genes which code for pathogen-derived resistance. See J. Sanford and S. Johnston, 113 J. Theor. Biol. 395 (1985); J. Sanford, 130 J. Theor. Biol. 469 (1988).

The polynucleic acid sequence includes a regulatory sequence upstream from, or 5' to, the gene. The regulatory sequence is positioned in the polynucleic acid sequence in operative association with the gene so as to be capable of inducing transcription of the gene. Regulatory sequences which may be used to provide transcriptional control of the gene in the polynucleic acid sequence are generally promoters which are operable in the target tissue cells. Exemplary promoters include, for example, the human α -actin promoter, see T. Miwa and L. Kedes, 7 Molec. Cell Biol. 2803 (1987), the human β -actin promoter, J. Leavitt et al., 4 Molec. Cell Biol. 1961 (1984), the troponin T gene promoter, see T. Cooper and C. Ordahl, 260 J. Biol. Chem. 11140 (1985), the human heat shock protein (HSP) 70 promoter, retrovirus long terminal repeats such as the Rous Sarcoma Virus long terminal repeat, see generally RNA Tumor Viruses (R. Weiss, N. Teich, H. Varmus and J. Coffin Eds. 2d ed. 1984), and the metallothionin gene promoter. The promoter and gene should be capable of operating in the cells, or cells of the tissue, to be transformed (i.e., the promoter should be capable of inducing transcription of the gene, and the gene should code for an mRNA sequence capable of being translated), with the requirements for operability known in the art. See generally R. Old and S. Primrose, Principles of Gene Manipulation (3d Ed. 1985). With respect to tissue, these elements need only be operable in one cell type in that tissue.

Other regulatory elements which may optionally be incorporated into the polynucleic acid sequence include enhancers, termination sequences, and polyadenylation sites,

-10-

as known in the art, as necessary to obtain the desired degree of expression of the gene in the cell into which it is inserted.

Any microprojectile acceleration cell transformation apparatus can be used in practicing the present invention, so long as the apparatus is modified as necessary for the treatment of air-breathing animals. Exemplary apparatus is disclosed in Sanford et al., Delivery of Substances into Cells and Tissues using a Particle Bombardment Process, 5 Particulate Science and Technology 27 (1988), in Klein et al., High-Velocity Microprojectiles for Delivering Nucleic Acids into Living Cells, 327 Nature 70 (1987), and in Agracetus European Patent Application Publication No. 0 270 356, titled Pollen-Mediated Plant transformation. We used a commercially available device from Biolistics, Inc., 108 Langmuir Laboratory, Cornell Business and Technology Park, Brown Road, Ithaca, NY, 14850. This device is designated a Model BPG-4 Particle Acceleration Apparatus and is configured essentially as described in Klein et al., 327 Nature 70 (1987). The device, illustrated in Figures 1 through 5 (with improvements shown in Figs. 2-5), comprises a bombardment chamber 10 which is divided into two separate compartments 11,12 by an adjustable-height stopping plate support 13. An acceleration tube 14 is mounted on top of the bombardment chamber. A macroprojectile 15 is propelled down the acceleration tube at stopping plate 16 by a gunpowder charge. A conventional firing mechanism 18 and evacuating apparatus 19 are provided. The stopping plate 16 has a bore hole 17 formed therein which is smaller in diameter than the macroprojectile, the macroprojectile carries the microprojectiles, and the macroprojectile is aimed and fired at the bore hole 17. When the macroprojectile 15 is stopped by the stopping plate 16, the microprojectiles are propelled through the bore hole 17. The target tissue 40, here schematically illustrated as an animal subject, is positioned in the bombardment chamber so that microprojectiles propelled through the bore hole 17 penetrate the cell membranes of the cells in the target tissue

-11-

and deposit DNA constructs carried thereon in the cells of the target tissue. The bombardment chamber 10 is partially evacuated prior to use to prevent atmospheric drag from unduly slowing the microprojectiles. The chamber is only partially evacuated so that the target tissue is not unduly desiccated during bombardment thereof. A vacuum of between about 20 to 26 inches of mercury is suitable.

Microprojectiles (i.e., microparticles) used in carrying out the present invention may be formed from any material having sufficient density and cohesiveness to be propelled into the cells of the tissue being transformed, given the particle's velocity and the distance the particle must travel. Non-limiting examples of materials for making microprojectiles include metal, glass, silica, ice, polyethylene, polypropylene, polycarbonate, and carbon compounds (e.g., graphite, diamond). Metallic particles are currently preferred. Non-limiting examples of suitable metals include tungsten, gold, and iridium. The particles should be of a size sufficiently small to avoid excessive disruption of the cells they contact in the target tissue, and sufficiently large to provide the inertia required to penetrate to the cell of interest in the target tissue. Gold particles ranging in diameter from about one micrometer to about three micrometers are preferred for in situ bombardment, and (more particularly) tungsten particles about one micrometer in diameter are preferred for in vitro bombardment of muscle.

The polynucleic acid sequence may be immobilized on the particle by precipitation. The precise precipitation parameters employed will vary depending upon factors such as the particle acceleration procedure employed, as is known in the art. The carrier particles may optionally be coated with an encapsulating agent such as polylysine to improve the stability of polynucleic acid constructs immobilized thereon, as discussed in EPO Application 0 270 356, at Column 8.

Skin in vertebrates is formed from an outer epidermis and an underlying dermis (or corneum). Further underlying the dermis there usually is a loose, spongy layer called the

-12-

hypodermis which is herein treated by definition as a part of the skin. The dermis and/or the hypodermis are the preferred tissue targets when the object of the transformation is to administer a protein or peptide to the animal subject in a manner which will evoke a physiological response thereto in the animal subject, as discussed above.

In land-dwelling vertebrates such as land-dwelling amphibians, reptiles, birds, and mammals, the epidermis is generally comprised of, from the outer surface to the inner surface, the following layers: (a) the stratum corneum, or horny layer, composed of thin squamous (flat) keratinized cells that are dead and continually being shed and replaced; (b) the stratum lucidum, or clear layer, in which keratinocytes are closely packed and clear, and in which the nuclei are absent and the cell outlines indistinct; (c) the stratum granulosum, or granular cell layer, where the process of keratinization begins; (d) the stratum spinosum, or prickle cell layer, where cells are rich in ribonucleic acid and thereby equipped to initiate protein synthesis for keratinization; and (e) the stratum basale, or basal cell layer, which is composed of a single layer of columnar cells that are the only cells in the epidermis that undergo mitosis. See generally G. Thibodeau, Anatomy and Physiology, 114-19 (1987); R. Frandson, Anatomy and Physiology of Farm Animals, 205-12 (2d Ed. 1981); R. Nickel et al., Anatomy of the Domestic Birds, 156-57 (1977).

The dermis, also called the "true skin," is generally composed of a stratum superficiale, or papillary layer, which immediately underlies the epidermis, and an underlying stratum profundum, or reticular layer. The arteries, veins, capillaries, and lymphatics of the skin are concentrated in the dermis. The reticular layer generally includes a dense network of interlacing white collagenous fibers, skeletal muscles, and involuntary muscles. The papillary layer is composed of loose connective tissue and a fine network of thin collagenous and elastic fibers.

-13-

The hypodermis, or superficial fascia, is a loose, spongy subcutaneous layer rich in fat, areolar tissue, and blood vessels. When skin is removed from an animal by blunt dissection, separation usually occurs in the cleavage plane that exists between the hypodermis and underlying tissues, with at least portions of the hypodermis thus adhering to the skin.

In the method of the present invention, dermis and epidermis may be transformed by either (a) propelling the microprojectiles through the epidermis, or (b) surgically exposing the hypodermis and dermis by incision and blunt dissection of a skin flap from the animal and propelling the microprojectiles directly into the hypodermis and dermis without projecting the microprojectiles through the outer surface layer, and then restoring the dissected skin flap to the position on the animal from which it came. The skin flap can remain attached to the animal for microprojectile bombardment or briefly removed for microprojectile bombardment and then grafted back to the animal. If removed from the animal the skin flap can be returned to the same or a different site on the animal, or can be transplanted to a different animal. We prefer to leave the skin flap attached. We have found greater transformation of the dermis by surgically exposing the dermis so that the microparticles need not pass through the epidermis, but have also found substantial transformation of the dermis even when the microparticles are propelled through the epidermis of land-dwelling vertebrates.

Various aspects of the present invention are explained in the examples which follow. These examples are given to illustrate the invention, and are not to be construed as limiting thereof.

EXAMPLE 1

Particle-Mediated Transformation of Terminally Differentiated Skeletal Myotubes

This example demonstrates that primary cultures of

-14-

fully differentiated, non-dividing skeletal myotubes can be transformed in vitro using a DNA-particle accelerator. The introduced genes are not rapidly degraded, but remain transcriptionally active over the life of the culture (twelve days).

Myoblast cultures (4×10^5 cells) were established from breast muscles of eleven-day chick embryos in gelatin-coated 60 mm plastic dishes in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 10% horse serum and 5% embryo extract. After five days without fresh media, cultures consisted almost entirely of multinucleated myotubes, some of which showed cross-striations. Some cultures were also treated with cytosine arabinoside (ara-C: 1.5 to 3.0 $\mu\text{g/ml}$) to inhibit growth of residual undifferentiated myoblasts or non-myogenic cells. See G. Paulath et al., Nature 337, 570 (1989). At this stage (five days in culture) the conditioned medium was removed and saved, and the plates were placed in a vacuum chamber. Tungsten microprojectiles (mean diameter 1 μm) were coated with pHB-LUC, a plasmid construct in which the firefly luciferase gene, J. de Wet et al., Molec. Cell Biol. 7, 725 (1987), is driven by the human B-actin promoter, J. Leavitt et al., Molec. Cell Biol. 4, 1961 (1984), a promoter which has strong constitutive activity in these cells. Each culture was bombarded under vacuum (twenty-nine inches Hg) with 2 μl of microprojectile suspension. The macroprojectile was started 3 cm from the top of the barrel and accelerated with a #1 gunpowder 22 caliber cartridge. The petri dish was placed at the bottom of the chamber. The device used and the methods for coating of the microprojectiles are described in J. Sanford et al., Particulate Sci. Technol. 5, 27 (1987) and in T. Klein et al., Nature 327, 70 (1987). In the present study, pilot experiments were performed to establish the particle velocity, particle size, particle composition, and cell density that resulted in maximal expression of luciferase activity following bombardment for our particular circumstances. Once these conditions were optimized, the experiments described in

-15-

Table 1 were performed. Whole cell lysates were prepared from cells two days after bombardment and luciferase activity was measured in a Berthold Biolumat LB9500C luminometer following addition of luciferin in the presence of excess ATP. J. de Wet et al., Molec. Cell Biol. 7, 725 (1987). These data are also shown in Table 1.

TABLE 1

Expression of firefly luciferase gene driven by the human β -actin promoter following transfection by microparticle bombardment of fully differentiated skeletal myotubes.

<u>Luciferase activity</u> <u>(peak, light emission/60 mm culture dish)</u>	
Mock transfection (n=3)	14 \pm 7
15 pHB-LUC (n=6)	112,164 \pm 19,086
pHB-LUC + ara-C (n=6)	107,620 \pm 19,881

Transformation by microprojectiles produced reporter gene activities that were 10-20X higher/plate and 200-400X higher/ μ g DNA than activities obtained by transformation of myotube cultures by standard calcium phosphate co-precipitation. See C. Chen and H. Okayama, Molec. Cell Biol. 7, 2745 (1987).

25

EXAMPLE 2

Fate Over Time of DNA Introduced in
Terminally Differentiated Skeletal
Myotubules by Particle Bombardment

This Example addressed the fate of the introduced DNA over time by measuring the response of an inducible promoter at varying intervals after bombardment. Myotubes were transformed as described in Example 1 above, but with the

-16-

firefly luciferase gene under the control of the human HSP70 promoter. See B. Wu et al., Proc. Nat. Acad. Sci. USA 83, 629 (1986). On days 2-7 following bombardment, luciferase activity was measured in sister cultures that were either maintained at 37°C (Control = C) or placed at 45°C for 90 minutes followed by recovery at 37°C for three hours (Heat Shock = HS).

The cultures maintained for 6-7 days following bombardment were re-fed at two day intervals with conditioned media (depleted of mitogenic growth factors) from untransfected myotube cultures. Figure 6 demonstrates that inducible expression of the introduced plasmid was maintained over this period. As calculated relative to the basal level expression in the control plates, there was no diminution in expression between day 2 (7.9-fold induction) and day 7 (11.9-fold induction) cultures. Thus, there was no substantial degradation of the plasmid or silencing of the heterologous promoter during the lifetime of these cultures.

EXAMPLE 3

Site of Transgene Expression in Cultures of Terminally Differentiated Myotubules

This Example was conducted to determine whether trans-gene expression was occurring within the fully differentiated myotubes, as distinguished from mononuclear cells that remain within these primary cultures. Cultures of differentiated myotubes were transfected, by microprojectile bombardment as described in Example 1, with a plasmid construct containing the Drosophila Alcohol Dehydrogenase (ADH) gene under the control of the Rous Sarcoma Virus long terminal repeat (pRSV-ADH). On the following day, cells were fixed and stained according to the method described by C. Ordahl et al., Molecular Biology of Muscle Development 547 (C. Emerson et al. Eds. 1986), and photographed under phase contrast with misaligned phase rings.

Although Drosophila ADH was detectable in mononuclear cells, the large majority of the activity was found within

-17-

multinucleated fused myotubes. Interestingly, two patterns of myotube staining were evident. In some myotubes, ADH activity was spatially limited around a single nucleus, while the remainder of the multinucleated cell was devoid of activity. This staining pattern suggests that conditions existed in these cells to restrict expression of the transgene to a spatial domain surrounding an individual nucleus. However, in other myotubes, ADH staining was distributed uniformly throughout the cell. This diffuse pattern of transgene expression implies either that multiple nuclei were transformed in a single myotube, or that, under some conditions, the ADH protein was free to diffuse throughout the entire span of these elongated cells. In view of the apparent frequency of transformation, the latter explanation is favored.

EXAMPLE 4

Transformation of Alternate

Cells with pRSV ADH

The experiment described in example 3 above was repeated in essentially the same manner, except that cardiac cells in vitro were used instead of skeletal myotubes. No positive results were seen. The lack of transformation was apparently due to the very few number of cells on the plate of cells used.

The experiment described in example 3 above was again repeated in essentially the same manner, except that whole mouse diaphragm was used instead of skeletal myotubes. The diaphragm was held flat on a dish with a piece of screen and the screen held down by weights. No transformation was seen. It appears that the 1 micron tungsten microparticles employed did not have sufficient kinetic energy to penetrate the diaphragm tissue.

EXAMPLE 5

Apparatus for Transformation of Animals

The device employed in the above examples was modified

-18-

for the transformation of tissue in whole animals in the manner illustrated in Figures 1 through 5. The door 20 on the bombardment chamber 10 was opened and an animal bombardment fitting, 30 or "trap," inserted. The animal bombardment fitting included a cover plate 31 and an animal chamber 32. The animal chamber 32 has a top wall, bottom wall, side walls, a back wall, and an outer flange 33. The chamber 32 is inserted through an opening in the cover plate and sealed thereto by means of a rubber gasket on the front side of the cover plate positioned between the cover plate opening and the edge of the animal chamber flange. Threaded fasteners 34 secure the animal chamber 32 to the cover plate 31. The back side of the cover plate has a rubber gasket spaced inwardly from the outer edge thereof for sealing the cover plate to the bombardment chamber 10.

The top of the animal chamber has an opening 35 formed therein which, when the animal chamber is installed in the bombardment chamber, is axially aligned with the center axis of the bore hole 17 of the stopping plate 16. An inner cylindrical sleeve 36, open at the top and bottom, is connected and sealed in the top opening 35. The bottom edge portion of the inner sleeve has a rubber gasket 37 inserted therein.

A sealing plate 38 is provided for sealing the bottom opening of the inner sleeve 36. The sealing plate has a center opening 39 formed therein. The surface of the tissue on the animal subject 40 to be transformed is placed in contact with the sealing plate 38 so that the tissue to be transformed is accessible through the center opening 39. The sealing plate 39 is then placed in contact with the bottom edge portion of the inner sleeve 36 and a vacuum drawn in the vacuum chamber. The contact of the subject tissue to the sealing plate 38, the sealing plate to the inner sleeve 36, the inner sleeve to the animal chamber 32, the animal chamber to the cover plate 31 and the cover plate to the bombardment chamber 10 all operate to seal the bombardment chamber 10. A screen is provided across the center opening of the sealing

-19-

plate on the bottom surface thereof to reduce the tendency of tissue to be drawn into the chamber. When the microprojectiles are accelerated, the opening 39 in the sealing plate 38 is positioned so that the microprojectiles contact the tissue surface accessible through the opening. A sponge or other spacing means 41 can be used to hold the animal subject up against the sealing plate.

EXAMPLE 6

Particle Bombardment of Euthanized Mice

Mice were euthanized and the hair removed from their hind legs with a depilatory (NEET™) to expose the skin on the hind legs. The skin was then either left in position or dissected away to expose underlying muscle. The animals were positioned in the apparatus described in example 5 above, either hind leg skin or muscle tissue positioned for bombardment, a vacuum of 26 inches of mercury drawn in the chamber, and the tissue bombarded with 1 micron tungsten microprojectiles. 1 micron tungsten particles were found too small to penetrate either muscle or skin. 3.4 micron tungsten particles were then tried, and were found to penetrate muscle and skin. Best were gold particles, 1 to 3 microns in diameter.

EXAMPLE 7

Particle Bombardment of Skin and Ear in Live Mice

Live adult female Balb C and Charles River CD1 mice were transformed by the apparatus and procedures described in the preceding examples. Gold particles 1 to 3 microns in diameter were coated with pHb-LUC by precipitation, as described above. Animals were anesthetized with a mixture containing equal parts of ketamine and xylazine (0.067 mg/g body weight). The target areas were hind leg skin and ear, which were prepared with a depilatory as described above. A vacuum of 26 inches of mercury was drawn for hind leg skin and 20 inches of mercury drawn for ear. After bombardment, the

-20-

tissue showed little or no evidence of damage. A faint brown stain was evident in the area containing particles in most animals and, rarely, a small ($<1\text{mm}^2$) area of intradermal hemorrhage from small blood vessels was noted. Peak luciferase activity of the skin and ears on day one after transfection in counts per minute is shown in Figure 7. The values are means \pm the standard deviation. The activity of 17 skin samples was $4,699 \pm 4,126$ and the activity of 12 ear samples was $47,114 \pm 3,679$. Photoluminescence was determined in duplicate on a Berthold LB 9500 C luminometer set for a ten second period of integration with 50 microliter (skin) and 25 microliter (ear) samples of extract. The mean luciferase activity for skin and ear over time in counts per minute is shown in Table 2 below.

TABLE 2

Luciferase Activities for Days 1 to 4

DAY:	1	2	3	4
Skin: mean	4699	810	217	104
s.d.	4126	1097	265	140
Ear: mean	149369	116114	69986	
s.d.	49392	122455	66461	

After recovery from anesthesia, animals showed no behavioral abnormalities and did not manifest evidence of pain or itching in the transformed area of skin. Histologic examination of bombarded skin revealed no significant alteration of tissue structure, and only occasional lymphocytes or polymorphonuclear leukocytes within the transformed area. In situ hybridization studies revealed a high proportion (approximately 25%) of cells within the epidermis that expressed luciferase mRNA, and a lower (but noticeable) proportion in the dermis and hair follicles.

-21-

EXAMPLE 8Local Transgene Activity in Ear of LiveMice by Particle Bombardment

Mouse ears were transformed with pGH precipitated on
5 1 to 3 gold microparticles as described in Example 7 above.
The plasmid pGH includes a human growth hormone (HGH) gene
driven by a metallothionin promoter. Local levels of HGH were
measured with a commercially available Nichols Institute
10 Allegro[™] HGH Radioimmunoassay. The RIA data is given in Table
3 below. Activity is expressed in counts per minute.

TABLE 3

<u>Local HGH Activity in Mouse Ear</u>		
	<u>Group</u>	<u>Activity</u>
15	Positive Control	441
	Left Ear	520
	Right Ear	220
	Negative Control	90
	Negative Control	67

20

The foregoing examples are illustrative of the present invention, and are not to be taken as limiting thereof. The invention is defined by the following claims, with equivalents of the claims to be included therein.

-22-

THAT WHICH IS CLAIMED IS:

1. A method of transferring a gene to preselected vertebrate cells, said method comprising the steps of:
 - (a) providing microprojectiles, the microprojectiles carrying polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the cells and a gene positioned downstream of the regulatory sequence and under the transcriptional control thereof; and
 - (b) accelerating the microprojectiles at the preselected cells, with the microprojectiles contacting the cells at a speed sufficient to penetrate the vertebrate cells and deposit the polynucleic acid sequences therein.
2. A method according to Claim 1, wherein the preselected cells are skin cells.
3. A method according to Claim 2, wherein the skin cells are selected from the group consisting of basal cells, dermis cells, and hypodermis cells.
4. A method according to Claim 1, wherein the preselected cells are muscle cells.
5. A method according to Claim 1, wherein the cells are transformed in vitro.
6. A method according to Claim 1, wherein said microprojectiles have diameters of from about 1 micron to about 3 microns.

-23-

7. A method of transferring a gene to a preselected vertebrate tissue, said method comprising the steps of:

(a) providing microprojectiles, the microprojectiles carrying polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the tissue and a gene positioned downstream of the regulatory sequence and under the transcriptional control thereof; and

(b) accelerating the microprojectiles at the preselected tissue, with the microprojectiles contacting the cells of the tissue at a speed sufficient to penetrate the tissue cells and deposit the polynucleic acid sequences therein.

8. A method according to Claim 7, wherein the preselected tissue is skin tissue.

9. A method according to Claim 7, wherein the skin tissue is selected from the group consisting of basal cell layer tissue, dermis tissue, and hypodermis tissue.

10. A method according to Claim 7, wherein the preselected tissue is muscle tissue.

11. A method according to Claim 7, wherein the tissue is transformed in vitro.

12. A method according to Claim 7, wherein said microprojectiles have diameters of from about 1 micron to about 3 microns.

-24-

13. A method of transferring a gene to a preselected vertebrate tissue in situ in a vertebrate subject, said method comprising the steps of:

- 5 (a) providing microprojectiles, the microprojectiles carrying polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the tissue and a gene positioned downstream of the regulatory sequence and under the transcriptional control thereof; and
- 10 (b) accelerating the microprojectiles at the vertebrate subject, with the subject positioned so that the microprojectiles contact the preselected tissue, and with the microprojectiles contacting the cells of the tissue at a speed sufficient to penetrate the tissue cells and deposit the polynucleic acid sequences therein.

14. A method according to Claim 13, wherein the preselected tissue is skin tissue.

15. A method according to Claim 13, wherein the skin tissue is selected from the group consisting of basal cell layer tissue, dermis tissue, and hypodermis tissue.

16. A method according to Claim 13, wherein the preselected tissue is muscle tissue.

17. A method according to Claim 13, wherein said microprojectiles have diameters of from about 1 micron to about 3 microns.

-25-

18. A method of administering a protein or peptide to a vertebrate subject, said method comprising the steps of:

- (a) selecting a target vertebrate tissue;
- (b) providing microprojectiles, the microprojectiles
5 carrying polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the selected tissue and a gene positioned downstream of the regulatory sequence and under the transcriptional control thereof, the gene coding for a protein or peptide;
- 10 (c) accelerating the microprojectiles at the selected target tissue, with the microprojectiles contacting the cells of the tissue at a speed sufficient to penetrate the tissue cells and deposit the polynucleic acid sequences therein to provide transformed tissue cells; and
- 15 (d) maintaining the transformed tissue in the animal subject, with the transformed tissue cells present in the subject in a number sufficient to produce a physiological response to the protein or peptide coded for by said gene in the subject upon expression of said gene.

19. A method according to Claim 18, wherein the transformed tissue cells produce an immune response to the protein or peptide coded for by said gene in the animal subject upon expression of said gene therein.

20. A method according to Claim 18, wherein the transformed tissue cells increase the systemic concentration of the protein or peptide coded for by said gene in the animal subject upon expression of said gene therein.

21. A method according to Claim 20, wherein said gene codes for the production of growth hormone.

-26-

22. A method according to Claim 18, wherein the selected target tissue is selected from the group consisting of dermis tissue and hypodermis tissue.

23. A method according to Claim 18, wherein the tissue is transformed in vitro, and the transformed tissue cells are thereafter transferred to the animal subject.

24. A method according to Claim 18, wherein the tissue cells are transformed in situ in the animal subject.

25. A method according to Claim 18, wherein said microprojectiles have diameters of from about 1 micron to about 3 microns.

-27-

26. A method of administering a protein or peptide to a vertebrate subject by in situ microprojectile bombardment, said method comprising the steps of:

5 (a) selecting a target vertebrate tissue residing in the subject, the tissue selected from the group consisting of dermis tissue and hypodermis tissue;

10 (b) providing microprojectiles, the microprojectiles carrying polynucleic acid sequences, the sequences comprising, in the 5' to 3' direction, a regulatory sequence operable in the selected tissue and a gene positioned downstream of the regulatory sequence and under the transcriptional control thereof, the gene coding for a protein or peptide;

15 (c) accelerating the microprojectiles at the subject, with the subject positioned so that the microprojectiles contact the selected target tissue, and with the microprojectiles contacting the cells of the target tissue at a speed sufficient to penetrate the tissue cells and deposit the polynucleic acid sequences therein to provide transformed tissue cells; and

20 (d) maintaining the transformed tissue cells in the subject, with the transformed tissue cells present in the subject in a number sufficient to produce a physiological response to the protein or peptide coded for by said gene in the subject upon expression of said gene.

27. A method according to Claim 26, wherein the transformed tissue cells produce an immune response to the protein or peptide coded for by the gene in the animal subject upon expression of said gene therein.

28. A method according to Claim 26, wherein the transformed tissue cells increase the systemic concentration of the protein or peptide coded for by said gene in the animal subject upon expression of said gene therein.

-28-

29. A method according to Claim 28, wherein said gene codes for the production of growth hormone.

30. A method according to Claim 26, wherein the microparticles are propelled through the epidermis of the animal subject.

31. A method according to Claim 26, further comprising the step of surgically exposing the tissue cells, and wherein the microparticles are propelled into the tissue cells without passing through the epidermis.

32. A method according to Claim 26, wherein said microprojectiles have diameters of from about 1 micron to about 3 microns.

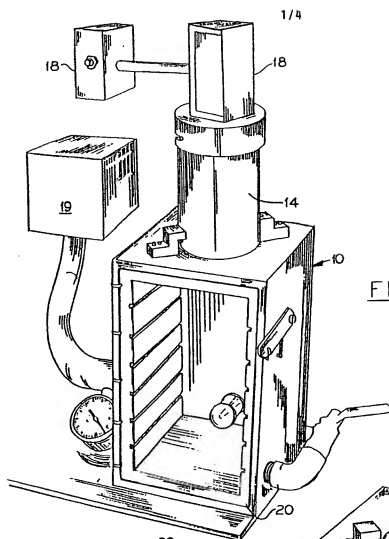


FIG. 1.

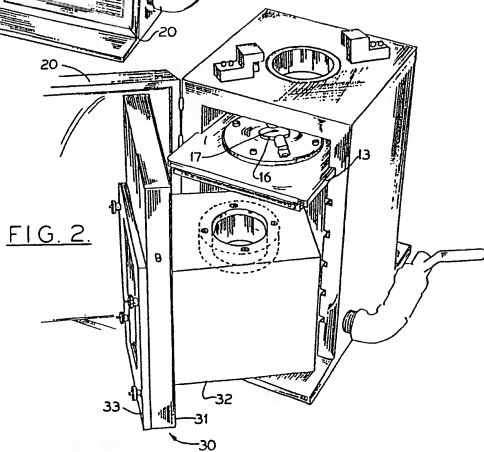


FIG. 2.

SUBSTITUTE SHEET

2/4

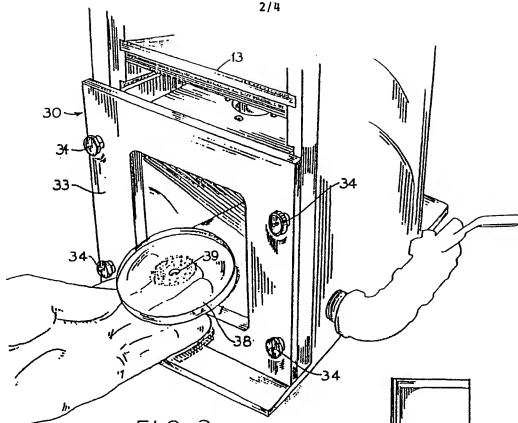


FIG. 3

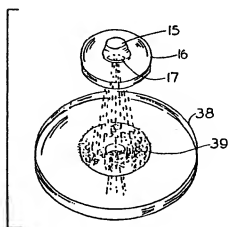


FIG. 5

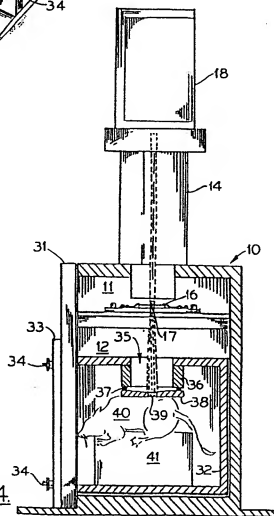
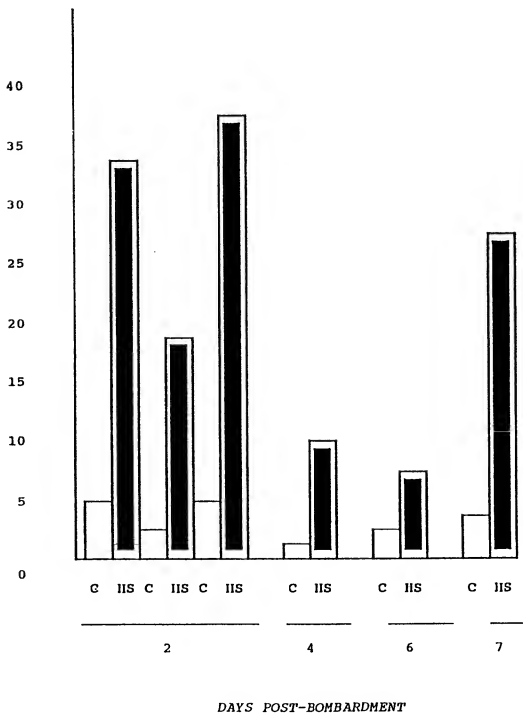


FIG. 4

SUBSTITUTE SHEET

3/4

FIGURE 6

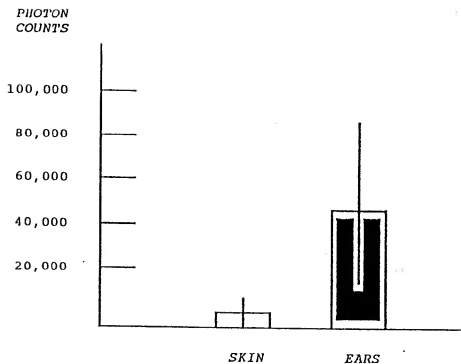


SUBSTITUTE SHEET

4/4

FIGURE 7

PEAK PHOTOLUMINESCENCE



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. CT/US90/06597

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC IPC(5):C12N 15/00 U.S. CL.: 800/2														
II. FIELDS SEARCHED Minimum Documentation Searched ? <table border="1"> <tr> <th>Classification System</th> <th>Classification Symbols</th> </tr> <tr> <td>U.S.</td> <td>435/172.3, 172.1, 69.1, 70.1 800/2</td> </tr> </table>			Classification System	Classification Symbols	U.S.	435/172.3, 172.1, 69.1, 70.1 800/2								
Classification System	Classification Symbols													
U.S.	435/172.3, 172.1, 69.1, 70.1 800/2													
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *														
III. DOCUMENTS CONSIDERED TO BE RELEVANT * <table border="1"> <tr> <th>Category *</th> <th>Citation of Document, ¹⁾ with indication, where appropriate, of the relevant passages ²⁾</th> <th>Relevant to Claim No. ³⁾</th> </tr> <tr> <td>X.P Y</td> <td>US. A. 4,945,000 (SANFORD, et al.) 31 July 1990. see entire document.</td> <td>1-17, 21-26 <u>28-30, 32</u></td> </tr> <tr> <td>X Y</td> <td>Particle Science & Technology, Vol. 5, issued 1987, SANFORD et al., "Delivery of Substances into cells and Tissues using a Particle Bombardment Process". pp. 27-37, see entire document.</td> <td>1,5-7 11-13, 17, 18, 24-26, <u>28-30, 32</u> 2-4, 8-10, 14-16, 19- 23, 27, 31</td> </tr> <tr> <td>X Y</td> <td>Federation of European Biochemical Societies, Vol. 244, No. 1, issued February 1989, ZELENIN, et al., "Genetic transformation of mouse cultured cells with the help of high velocity mechanical DNA injection" pp. 65-67, see entire document.</td> <td>1,5,6,11, <u>12,17,25,32</u> 7,8-10, 13-16, 18-24</td> </tr> </table>			Category *	Citation of Document, ¹⁾ with indication, where appropriate, of the relevant passages ²⁾	Relevant to Claim No. ³⁾	X.P Y	US. A. 4,945,000 (SANFORD, et al.) 31 July 1990. see entire document.	1-17, 21-26 <u>28-30, 32</u>	X Y	Particle Science & Technology, Vol. 5, issued 1987, SANFORD et al., "Delivery of Substances into cells and Tissues using a Particle Bombardment Process". pp. 27-37, see entire document.	1,5-7 11-13, 17, 18, 24-26, <u>28-30, 32</u> 2-4, 8-10, 14-16, 19- 23, 27, 31	X Y	Federation of European Biochemical Societies, Vol. 244, No. 1, issued February 1989, ZELENIN, et al., "Genetic transformation of mouse cultured cells with the help of high velocity mechanical DNA injection" pp. 65-67, see entire document.	1,5,6,11, <u>12,17,25,32</u> 7,8-10, 13-16, 18-24
Category *	Citation of Document, ¹⁾ with indication, where appropriate, of the relevant passages ²⁾	Relevant to Claim No. ³⁾												
X.P Y	US. A. 4,945,000 (SANFORD, et al.) 31 July 1990. see entire document.	1-17, 21-26 <u>28-30, 32</u>												
X Y	Particle Science & Technology, Vol. 5, issued 1987, SANFORD et al., "Delivery of Substances into cells and Tissues using a Particle Bombardment Process". pp. 27-37, see entire document.	1,5-7 11-13, 17, 18, 24-26, <u>28-30, 32</u> 2-4, 8-10, 14-16, 19- 23, 27, 31												
X Y	Federation of European Biochemical Societies, Vol. 244, No. 1, issued February 1989, ZELENIN, et al., "Genetic transformation of mouse cultured cells with the help of high velocity mechanical DNA injection" pp. 65-67, see entire document.	1,5,6,11, <u>12,17,25,32</u> 7,8-10, 13-16, 18-24												
* Special categories of cited documents: * "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claims or which is cited to establish the public state of the art or justify citation of other special features (as searching) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such docu- ments, such combination being obvious to a person skilled in the art "&" document member of the same patent family														
IV. CERTIFICATION <table border="1"> <tr> <td>Date of the Actual Completion of the International Search</td> <td>Date of Mailing of this International Search Report</td> </tr> <tr> <td>12 February 1991</td> <td>08 MAR 1991</td> </tr> <tr> <td>International Searching Authority</td> <td>Signature of Authorized Officer</td> </tr> <tr> <td>ISA/US</td> <td><i>Kimberlin M. Toobey</i> Kimberlin M. Toobey</td> </tr> </table>			Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	12 February 1991	08 MAR 1991	International Searching Authority	Signature of Authorized Officer	ISA/US	<i>Kimberlin M. Toobey</i> Kimberlin M. Toobey				
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report													
12 February 1991	08 MAR 1991													
International Searching Authority	Signature of Authorized Officer													
ISA/US	<i>Kimberlin M. Toobey</i> Kimberlin M. Toobey													

Form PCT/ISA210 (second sheet) (Nov. 11-87)

ebw

THIS PAGE BLANK (USPTO)